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Author/s:

Adams, LM;Simmons, CP;Rezmann, L;Strugnell, RA;Robins-Browne, RM

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Identification and Characterization of a K88- and CS31A-Like Operon of a Rabbit Enteropathogenic *Escherichia coli* Strain Which Encodes Fimbriae Involved in the Colonization of Rabbit Intestine

L. M. ADAMS,^{1,2} C. P. SIMMONS,² L. REZMANN,¹ R. A. STRUGNELL,² AND R. M. ROBINS-BROWNE^{1,2*}

Microbiological Research Unit, Department of Microbiology and Infectious Diseases, Royal Children's Hospital,¹ and Department of Microbiology and Immunology, University of Melbourne,² Parkville, Victoria 3052, Australia

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Initiation of attaching-effacing lesions, which characterize infections with rabbit enteropathogenic *Escherichia coli* (REPEC), requires bacteria to adhere to the intestinal epithelium. This adherence is reflected in vitro by the affinity of these *E. coli* strains for various types of eukaryotic cells. *TnphoA* mutants of REPEC 83/39 (O15:H-) which had lost the ability to adhere to HEp-2 epithelial cells, guinea pig ileal brush borders, and mouse erythrocytes were generated. DNA sequencing of the region surrounding the inactivating transposon insertions within a 95-kb plasmid, designated pRAP for REPEC adherence plasmid, revealed extensive homology between that region and the structural genes of enterotoxigenic *E. coli* operons encoding the K88 and CS31A fimbrial adhesins and the genes for the *afr2* adhesin from REPEC B10 (O103:H2). Seven genes of the *ral* operon (for REPEC adherence locus), including three putative minor fimbrial subunit genes (*ralC*, *ralF*, and *ralH*), a major fimbrial subunit gene (*ralG*), a gene of unknown function (*rall*), and genes for two fimbrial subunit chaperones (*ralD* and *ralE*), were sequenced. When inoculated perorally into weanling rabbits, a mutant with a *TnphoA* insertion in the *ralE* gene showed a 10-fold reduction in colonizing ability, with only 1 of 10 rabbits excreting bacteria compared to all 5 of those infected with the wild-type parent strain ($P = 0.002$). The severity of the diarrheal illness caused by the mutant strain was also reduced. Western blotting of surface protein extracts of strain 83/39 with hyperimmune anti-83/39 antiserum, adsorbed with the *ralE* mutant, revealed a 32-kDa protein which was absent from protein extracts of two nonadherent mutants. The adsorbed antiserum also bound to the surface of strain 83/39 but not to nonadherent mutants, as detected by immunogold labeling. These results indicate that the *ral* operon of REPEC 83/39 contains genes necessary for the biosynthesis of fine fimbriae which are responsible for in vitro adherence of the bacteria and play a role in their colonization of, and hence virulence for, rabbits. The putative major fimbrial subunit is a protein with an observed molecular size of approximately 32 kDa which, when assembled, appears to form a capsule of fimbriae surrounding the bacterium similar to that described for CS31A.

Strains of diarrheagenic *Escherichia coli* may be subdivided into at least five pathotypes by the virulence factors they harbor (35). Two of these pathotypes are enterotoxigenic *E. coli* (ETEC), which is characterized by its production of heat-labile and/or heat-stable enterotoxins and adhesive fimbriae, and enteropathogenic *E. coli* (EPEC) (35). The mechanism by which EPEC causes diarrhea is unclear since it does not produce any identifiable toxins and is not invasive per se (18, 34). An important part of the disease process involves the formation of distinctive attaching-effacing lesions in the small intestine (26, 46).

Attachment-effacement is a multistep process which requires an initial interaction of bacteria with the microvillus brush border followed by intimate attachment to the gut epithelium and effacement of microvilli with a concomitant triggering of a cascade of signal transduction events (8). The initial stage of adherence can be assessed in vitro by measuring bacterial adherence to HEp-2 cell monolayers (41). Virulent EPEC strains exhibit a pattern of localized adherence in vitro

which is mediated by a plasmid-encoded member of the type 4 family of fimbriae, named bundle-forming pili (11).

Rabbit EPEC (REPEC) bacteria constitute a subset of the EPEC pathotype because they have many of the major characteristics of human EPEC bacteria, such as the ability to cause severe diarrhea and to evoke attaching-effacing lesions in the absence of detectable toxins or significant epithelial invasion (6, 37). REPEC differs from human EPEC, however, in terms of its host specificity and its means of initial adherence. Thus, the in vitro adherence patterns of REPEC are more variable than those of human EPEC strains and, to date, no REPEC strain harboring the *bfp* genes responsible for the localized adherence of human EPEC has been identified (38).

A fimbrial adhesin mediating adherence of the REPEC prototype strain, RDEC-1 (O15:H-), to microvillus brush borders derived from rabbit ileum has been identified (7, 50). This adhesin, known as AF/R1 (adherence factor/rabbit 1), is plasmid encoded but has not been detected in any other REPEC strain (31, 38). More recently, a second, chromosomally encoded adhesin of REPEC has been identified in strain B10 (O103:H2) and has been designated AF/R2 (9). Two genes involved in the synthesis of this adhesin have been sequenced and were found to be homologous to subunit genes of the *fae* operon which encodes the K88 fimbriae of porcine ETEC and the *clp* operon which is responsible for expression of CS31A

* Corresponding author. Mailing address: Department of Microbiology and Infectious Diseases, Royal Children's Hospital, Parkville, Victoria 3052, Australia. Phone: (61-3) 9345-5741. Fax: (61-3) 9345-5764. E-mail: rbrowne@cryptic.rch.unimelb.edu.au.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
<i>E. coli</i>		
83/39	Wild-type REPEC, rifampin resistant	29
83/39/23	83/39, <i>ralE::TnphoA</i>	This study
83/39/51	83/39, <i>ralF::TnphoA</i>	This study
SM10 λ pir	<i>TnphoA</i> donor strain carrying plasmid pRT733	44
DH1	Laboratory strain, F ⁻ <i>endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1</i> λ ⁻ <i>recA1 gyrA96 relA1?</i>	13
Plasmids		
pRT733	Suicide plasmid vector (ampicillin resistant) carrying <i>TnphoA</i>	44
pRAP	95-kb REPEC adherence plasmid from <i>E. coli</i> 83/39 carrying the <i>ral</i> operon	This study
pWin	pBluescript KS containing the 8.3-kb <i>Bam</i> HI fragment of pRAP which includes the region from <i>ralC</i> to bp 678 of <i>ralG</i>	This study
p39CC	pBluescript KS containing the 3.3-kb <i>Cla</i> I fragment of pRAP which includes the region from bp 679 of <i>ralG</i> to <i>ralI</i>	This study

fimbriae by bovine ETEC strains (20, 22). Besides the four genes that encode fimbrial subunits (C, F, G, and H), these ETEC fimbrial operons contain two genes encoding subunit chaperones (D and E), a regulatory region incorporating two genes (A and B), and one or two open reading frames (ORFs) of unknown function (*faeI*, *clpL*, and *faeJ*) (references 1–3, 14, 22–25, 28, and 42 and GenBank accession no. L05182). The adhesive properties of these fimbriae are conferred by the major fimbrial subunit (G) rather than the secondary subunits (15).

In this study, we describe an operon responsible for the in vitro adherence of REPEC 83/39 (O15:H–). This operon contains homologs of all of the expressed structural genes of the *fae* operon. We also provide evidence that the fimbrial product of this operon plays a role in the virulence of strain 83/39 for rabbits.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. REPEC 83/39 has been described previously (29, 37, 38). Rifampin resistance was induced in this strain to allow selection during the bacterial mating procedure and to facilitate retrieval of the bacteria from the feces of infected rabbits. To this end, a heavy inoculum of strain 83/39 was incubated in 10 ml of Luria broth (LB) containing 100 μ g of rifampin (Boehringer GmbH, Mannheim, Germany) per ml at 37°C overnight with vigorous shaking. A sample of the resultant growth was plated onto Luria agar (LA) containing 50 μ g of rifampin per ml and tested with specific antiserum to confirm its identity as strain 83/39. The in vitro adherence properties of the rifampin-resistant derivative were unchanged compared to those of the parent strain reported previously (38).

For general purposes, bacteria were grown in LB at 37°C overnight with agitation or on LA overnight at 37°C. For experiments requiring expression of adhesins, bacteria were grown in Penassay broth (PAB; antibiotic medium 3 [Difco Laboratories, Detroit, Mich.]) at 37°C overnight without shaking.

***TnphoA* mutagenesis.** *TnphoA* mutants of 83/39 were derived by conjugating it with *E. coli* SM10 λ pir(pRT733), which carries the kanamycin resistance-encoding transposon, *TnphoA*, on a suicide plasmid (44). Matings were performed by spreading 20 μ l from a turbid suspension of each strain on an LA plate and incubating it at 37°C for 6 h. The plate was then washed with isotonic saline, and dilutions of the resultant suspension were plated on LA containing rifampin (50 μ g/ml), kanamycin (100 μ g/ml; Boehringer), 5-bromo-4-chloro-3-indolyl phosphate (XP, 50 μ g/ml; Sigma Chemical Co., St. Louis, Mo.) and glucose (0.2%). Mutants with in-frame transposon insertions into genes encoding extracytoplasmic proteins, which resulted in the formation of active alkaline phosphatase fusion proteins, were identified by their blue colony coloration in the presence of XP. Native alkaline phosphatase activity was suppressed by the glucose in the medium (44). Cointegrates were excluded by patch testing for resistance to ampicillin (50 μ g/ml; CSL, Melbourne, Victoria, Australia). The remaining blue colonies were examined for loss of adherence to HEp-2 cells.

HEp-2 cell adherence assays. Qualitative assays of adherence were performed by the method of Vial et al. (48). Quantitative adherence of *E. coli* to HEp-2 cells was assessed as described previously by Robins-Browne et al. (36).

DNA manipulation and sequencing. Plasmid DNA was extracted by a modification of the method of Birnboim and Doly (40) and analyzed, with or without

restriction digestion as appropriate, on 0.7% agarose gels. DNA was restricted with *Bam*HI, *Bgl*II, *Cla*I, and *Hpa*I endonucleases (Promega, Madison, Wis.) and, when required, cloned into the vector pBluescript KS (Stratagene, La Jolla, Calif.) by the procedures specified by the manufacturers.

To construct DNA probes, relevant fragments were excised from an agarose gel and extracted with a Bandpure DNA purification kit (Progen, Darra, Queensland, Australia) and then labeled with digoxigenin-11-dUTP (Boehringer) as described in the manufacturer's instructions. Target DNA was transferred to Zeta Probe nylon membrane (Bio-Rad Laboratories, Hercules, Calif.) by the alkaline blotting method as directed, after which hybridization and colorimetric development were carried out as specified for digoxigenin-11-dUTP probes. The presence of *TnphoA* was verified by hybridization with a 2.8-kb *Bgl*II fragment spanning the central region of the transposon. Other probes were derived from cloned strain 83/39 DNA as described below. Plasmid DNA was electroporated into DH1 or nonadherent mutants of strain 83/39 with a Bio-Rad gene pulser as described in the manufacturer's instructions.

Sequencing reactions were prepared with the ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Foster City, Calif.) and run on an Applied Biosystems 373A automated sequencer. DNA sequences were translated and analyzed by use of the suite of programs available through the Australian National Genomic Information Service. Percentage protein homologies between *ral* and homologous operons were calculated from amino acid sequence alignments as follows:

$$\% \text{ identity} = 2 \times \frac{\text{no. of identical aa}}{\text{no. of aa in Ral protein} + \text{no. of aa in homologous protein}} \times 100$$

$$\% \text{ similarity} = 2 \times \frac{\text{no. of identical and conserved aa}}{\text{no. of aa in Ral protein} + \text{no. of aa in homologous protein}} \times 100$$

where aa is amino acids.

Production of hyperimmune antiserum. A rabbit which had recovered from oral infection with strain 83/39 and was no longer excreting this strain was inoculated with increasing doses of 10⁴ to 10⁸ formalin-killed strain 83/39 via the ear vein twice weekly. Bacteria for injection were grown in PAB, inactivated in 3% formalin for 2 h, and washed three times in phosphate-buffered saline (pH 7.4; PBS). Two weeks after the final dose, a similar course of live, washed bacteria was administered at weekly intervals. Three weeks after the last injection, the rabbit was killed by exsanguination. The resultant hyperimmune serum was adsorbed three times against the nonadherent mutant 83/39/23 and then diluted for use in Western blotting or immunogold labeling as required.

Immunoblotting. To prepare crude fimbrial extracts, a 1-ml pellet of bacteria cultured in PAB was resuspended in 0.5 ml of 50 mM Na₂PO₄-2 M urea (pH 9.3) and incubated at 65°C for 20 min. Fimbriae were precipitated from the supernatant in 9 volumes of methanol at room temperature for 15 min and then recovered by centrifugation. Surface proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% acrylamide gels by the method of Laemmli (17) with a Novex (San Diego, Calif.) Xcell mini cell as directed by the manufacturer and then examined by immunoblotting with the adsorbed hyperimmune antiserum (45).

Immunogold labeling. Bacteria were cultured in PAB and washed once in PBS. Ten microliters of this suspension was loaded onto a 3-nm carbon-coated copper grid (Ted Pella Inc., Redding, Calif.) and left for 2 to 3 min. Excess bacterial suspension was removed by blotting, and the grid was floated for 15 min on a drop of hyperimmune serum diluted in PBS containing 1% bovine serum albumin and 1% Tween 20. The grid was then washed 10 times in the same diluent, with blotting performed between consecutive washes. It was then incubated for 15 min with a drop of a suspension of diluted 5-nm gold particles conjugated to anti-rabbit immunoglobulin G antibodies (Sigma). After five fur-

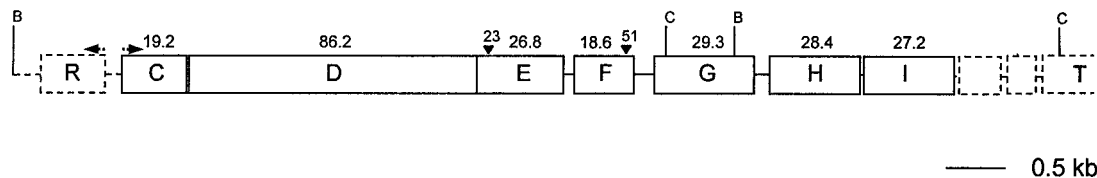


FIG. 1. Schematic diagram of the *ral* operon (drawn to scale). Numbers above the genes indicate the calculated molecular sizes of the predicted precursor polypeptides in kilodaltons. The sites of transposon insertion in the two nonadherent mutants are indicated by arrowheads, with the designation of the mutant placed above (e.g., 23 represents 83/39/23). The boxes delineated by broken lines represent potential ORFs. The *afiR* and *tsh* homologs are labeled R and T, respectively. The arrows indicate the probable directions of transcription. The *Bam*HI (B) and *Cla*I (C) restriction sites used for cloning are also shown.

ther washes with diluent and five with PBS, the sample was negatively stained with 1% ammonium molybdate. Stained preparations were viewed with a Philips 400 transmission electron microscope at an accelerating voltage of 60 kV.

Bacterial colonization of rabbits. To establish a role for the *ral* operon in vivo, 5- to 6-week-old New Zealand White rabbits were inoculated perorally with either strain 83/39 (5 rabbits) or strain 83/39/23 (10 rabbits). For these studies, bacteria were grown in PAB and then washed and resuspended in PBS. Prior to inoculation, rabbits received 2 ml of 5% (wt/vol) filter-sterilized sodium bicarbonate via a gastric tube. After 15 min, 2 ml of the appropriate bacterial suspension containing 6×10^6 CFU was administered in the same manner.

Infected rabbits were monitored daily for 3 weeks for weight loss and evidence of diarrhea such as loose stools and soiling of the hind limbs. At the end of the observation period, diarrheic rabbits were killed by an intravenous injection of sodium pentobarbitone. Bacterial excretion was determined semiquantitatively by culturing rectal swabs daily for the first 14 days, by which time an obvious difference in the comparative colonizing abilities of the wild-type and mutant strains was established. Plain sterile cotton-tipped swabs were inserted approximately 2 cm through the anus. Fecal material on the swabs was emulsified in 0.5 ml of PBS and plated on MacConkey agar supplemented with rifampin (50 μ g/ml), which precluded growth of all nonchallenge bacteria. The reliability of this counting method was established in other studies in which it was shown that the numbers of CFU obtained in this way reflected the actual CFU per 0.01 g of colon contents measured at autopsy (1). Bacteria isolated from rabbits infected with the mutant strain were checked periodically for resistance to kanamycin to ensure that no cross-contamination from rabbits infected with the wild-type bacteria had occurred. A rabbit was considered to be colonized when bacterial excretion was detected on 3 consecutive days.

Nucleotide sequence accession number. The GenBank accession number for the sequence reported in this paper is U84144.

RESULTS

Derivation of nonadherent mutants of *E. coli* 83/39 and identification of an 8.3-kb *Bam*HI fragment involved in adherence. *TnphoA* mutants of 83/39 were examined for their adherence to HEp-2 cells. Nonadherent mutants were also assessed for their ability to adhere to microvillus brush borders derived from the ileum of guinea pigs and to cause hemagglutination of murine erythrocytes (38). Mutants deficient in one type of adherence were invariably deficient in all three types (data not shown). Two mutants, designated 83/39/23 and 83/39/51, were chosen for further characterization.

Southern blot analysis of plasmids extracted from these strains, utilizing the probe derived from *TnphoA*, indicated that both mutants harbored a single transposon inserted in a ca. 95-kb plasmid called pRAP (for REPEC adherence plasmid). Since strain 83/39 carries three large plasmids, we decided to isolate the 95-kb plasmid to expedite further analysis and cloning of relevant regions. *E. coli* DH1 was transformed by electroporation with plasmid extracts from the two mutants. Attainment of DH1 transformants containing only the plasmid of interest was verified by examination of their plasmid profiles for a single plasmid of appropriate size which conferred kanamycin resistance and hybridized with the probe from *TnphoA* (data not shown). The unique *Bam*HI restriction site within *TnphoA* was then exploited to allow cloning of the regions upstream and downstream of the transposon insertion in each mutant. Analysis of these subclones showed that the transposon insertions were located 1.1 kb apart toward the 3' end of an

8.3-kb *Bam*HI fragment (Fig. 1). Initial sequencing of DNA surrounding the *TnphoA* insertions revealed regions with significant levels of homology (approximately 70 and 45% amino acid identities, respectively) to the products of the *faeE* and *faeF* genes of the K88 operon and the corresponding *clpE* and *clpF* genes of the CS31A operon of ETEC.

Cloning and characterization of the *ral* operon. To facilitate DNA sequencing, the 8.3-kb *Bam*HI fragment from parent strain 83/39 was cloned into pBluescript KS, thus creating pWin. A DNA probe consisting of a 1.8-kb *Hpa*I-*Cla*I fragment which included 0.2 kb of terminal *TnphoA* sequence and 1.6 kb of DNA downstream of the transposon insertion site in mutant 83/39/23 was used to confirm that pWin contained the correct fragment. When pWin was introduced into either of the nonadherent mutants, the adherence phenotypes on all cell types were restored, although not to wild-type levels (Table 2 and data not shown). The operon contained by pWin, which is required for in vitro adherence, was designated *ral* for REPEC adherence locus, and individual genes were named after their *fae* homologs.

Sequencing of the 3' end of pWin yielded homology (approximately 33% amino acid identity after translation) to internal 3' sequences of *faeG* and *clpG*, which encode the major fimbrial subunits of K88 and CS31A, respectively, indicating the probability that further *ralG* sequence and *ral* operon genes would be found downstream from the 8.3-kb *Bam*HI fragment. Hence, a 0.6-kb *Cla*I-*Bam*HI fragment from pWin incorporating the 5' end of *ralG* (Fig. 1) was used to probe *Cla*I digests of pRAP. A 3.3-kb probe-positive *Cla*I fragment was then cloned into pBluescript KS to create p39CC.

The sequence of the *ral* operon is shown in Fig. 2. Analysis of this sequence revealed seven genes, which were designated *ralC* to *ralI* in keeping with homologous genes from the *fae* operon. The arrangement of these genes is shown in Fig. 1. Data regarding the length of the ORFs, the number of amino acid residues in the proposed polypeptide, the predicted mo-

TABLE 2. Adherence of nonadherent mutants complemented with pWin to HEp-2 epithelial cells

<i>E. coli</i> strain	Characteristic	% Adherence ^a	
		Background	With pWin
83/39	Wild type	100	NT ^b
83/39/23	<i>ralE</i> mutant	0.8 \pm 0.3	45 \pm 35 ^c
83/39/51	<i>ralF</i> mutant	0.9 \pm 0.6	73 \pm 54 ^c
DH1	Laboratory strain	0.4 \pm 0.4	0.2 \pm 0.1

^a As a percentage of the adherence of strain 83/39. Values are means \pm standard deviations of at least three independent observations.

^b NT, not tested.

^c Significantly greater than the value for uncomplemented mutant ($P < 0.05$, Student's *t* test).



FIG. 2. Sequence of the *ral* operon showing the seven ORFs designated *ralC* to *ralI* with the amino acid translation shown above the nucleotide sequence. Initiation and stop codons are in bold type; potential ribosome binding sites are underlined.

lular cases of these genes and their relatedness to products of homologous genes to genes of other operons are summarized in Table 3. Although there was significant homology between *RalG* and *AF/R2*, homology between *RalG* and *AfrA*, the major fimbrial subunit of *AF/R1*, was very low. *AfrA* is more than 100 amino acids shorter than *RalG* and had only 21% amino acid identity (37% similarity)

when aligned with the total of the first 166 amino acids of *RalG*. With the extra length of *RalG* was taken into account, the percentage amino acid identity declined to 16% (27% similarity). This suggests that the two proteins are unrelated. In contrast, three ORFs from a *Salmonella gallinarum* virulence plasmid which have been sequenced recently (GenBank accession no. AF005899) exhibit amino acid identities ranging from 27 to

TABLE 3. Characteristics of the genes of the *ral* operon and their relatedness to products of homologous genes from the *fae*, *clp*, and *afr2* operons

<i>ral</i> gene	Gene length (bp)	No. of amino acid residues	Calculated molecular size (kDa)	% Homology to analogous fimbrial proteins of ^a :					
				K88		CS31A		AF/R2	
				I	S	I	S	I	S
<i>ralC</i>	549	182	19.2	61.2	76.0	61.3	76.8		
<i>ralD</i>	2,424	807	86.2	67.1	81.8				
<i>ralE</i>	723	240	26.8	71.9	85.5	71.2	85.5		
<i>ralF</i>	513	170	18.6	48.1	66.7	46.9	67.9		
<i>ralG</i>	843	280	29.3	33.3	55.9	34.1	57.7	68.7	79.8
<i>ralH</i>	783	260	28.4	60.6	77.3	60.9	78.3	91.2	94.2
<i>ralI</i>	768	255	27.2	50.7	68.4	69.1	68.4		

^a Percentage amino acid identities (I) and similarities (S) were calculated from alignments as described in Materials and Methods. Protein sequences for comparison were either extracted from the SwissProt database or translated from nucleotide sequences submitted to GenBank. Accession numbers from the SwissProt database were P04738, P06970, P25401, P25447, P02970, P33782, P33783, and Q05433; those from GenBank were L48184, M96174, M59905, M96152, L05182, and U77302.

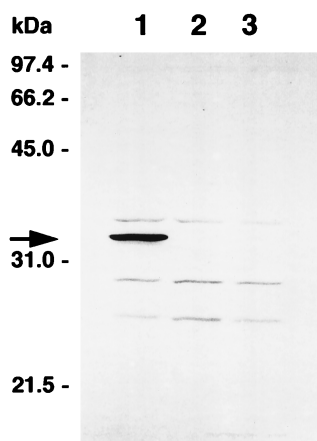


FIG. 3. Immunoblot of crude fimbrial extracts of REPEC 83/39 and two nonadherent mutants blotted with hyperimmune antiserum raised against 83/39 and then adsorbed with 83/39/23. The arrow indicates a protein of approximately 32 kDa produced by the wild type which is absent from preparations of the nonadherent mutants. Lanes: 1, 83/39; 2, 83/39/23; 3, 83/39/51.

60% (68 to 80% similarity) to large parts of the RalG, RalH, and RalI proteins.

Evidence that *ral* encodes a surface structure with a 32-kDa subunit. In an effort to identify the major structural subunit of the adhesin, Western blots of crude fimbrial extracts of strain 83/39 were probed with hyperimmune anti-83/39 antiserum that had been adsorbed extensively with the *ralE* mutant strain, 83/39/23. By this approach, a protein of approximately 32 kDa was identified in extracts of 83/39; this protein was absent from extracts of 83/39/23 and 83/39/51 (Fig. 3).

Despite several attempts to visualize fimbriae on strain 83/39 by use of transmission electron microscopic examination of negatively stained samples, no surface structures were observed. Consequently, the adsorbed antiserum was used for immunogold labeling of 83/39 and the nonadherent mutants in experiments designed to visualize any surface appendages. The results (Fig. 4) indicated that the 32-kDa protein was surface associated, and although no obvious protrusions were observed, the antiserum bound to the surface of more than 95% of 52 wild-type bacteria to form a halo, suggesting the presence of a network of fine fimbriae (Fig. 4A). Confirmation that this pattern resulted from binding of antibodies to the 32-kDa protein and not a cross-reacting species was obtained by demonstrating the complete absence of gold particles from all of eight *ralE* mutant cells and six *ralF* mutant cells ($P < 0.0001$ for both; Fisher's exact test) (Fig. 4B). The numbers of mutant cells observed were lower than those of the wild type since they were easily removed by washing during the staining procedure for electron microscopy.

The *ral* operon is important for colonization in vivo. Given that transposon insertions within the *ral* operon abrogated bacterial adherence in vitro, we determined if Ral also contributed to bacterial colonization of rabbit intestine. Of the five rabbits inoculated with wild-type 83/39, three began excreting bacteria within 24 h of inoculation. This number rose to all five rabbits by day 4 after infection. The number of bacteria recovered from rectal swabs increased steadily during the sampling period, peaking at 10^9 CFU/swab in three of five rabbits, which experienced severe diarrhea accompanied by substantial weight loss (Fig. 5). This contrasts to the group inoculated with the nonadherent *ralE* mutant, 83/39/23, in which only 1 of 10 rabbits was colonized, with a maximum swab count reaching

only 10^6 CFU (Fig. 5). The difference in the colonizing ability of the two strains was highly significant ($P = 0.002$; Fisher's exact test).

In general, rabbits with counts of $\geq 10^6$ CFU developed diarrhea, including the rabbit colonized by the mutant, in which a mild self-limiting illness was observed. Although this rabbit also experienced some weight loss (up to 3% of its body weight in 24 h) while it was diarrheic, the weight loss was much less pronounced than that in rabbits with diarrhea induced by the wild-type strain. These animals experienced prolonged periods of illness during which weight loss was sometimes as rapid as 7% of total body weight in 24 h (Fig. 6). These findings indicate that 83/39/23 retained the ability to cause diarrhea once it became established in the intestine, but its capacity to induce severe illness appeared to be severely compromised, probably due to its inability to colonize the intestine as efficiently as the wild type.

DISCUSSION

Full expression of virulence by EPEC strains of human or rabbit origin requires specific fimbriae which mediate attachment of the bacteria to the intestinal tract (19, 50). In this study, we describe an operon homologous to those encoding K88 and CS31A fimbriae from ETEC in a rabbit-specific strain of EPEC. This operon, which we termed *ral*, contains genes essential for the biosynthesis of a fimbrial adhesin with a putative 32-kDa major subunit. The adhesin mediates adherence of strain 83/39 in vitro and is important in mediating colonization of the rabbit host.

The overall arrangement of the *ral* operon closely resembles that of the *fae* and *clp* operons, with homologous genes occupying the same relative position in each operon. The *ral* operon also has some of the more specific features of the *fae* operon such as the overlapping reading frames of the two chaperone genes and the apparent absence of promoters within the region carrying the structural genes (22, 23). This general similarity together with the significant levels of homology exhibited by individual genes makes it reasonable to propose functions for the *ral* gene products based on the known roles of their Fae counterparts. Thus, we propose that RalC, RalF, and RalH are minor fimbrial subunits of the fimbrial structure which is primarily composed of RalG, the major fimbrial subunit. The effective transport and correct assembly of the subunits are likely to depend on the action of the periplasmic chaperone, RalE, and the outer membrane chaperone, RalD. Since there is no known function for FaeI (3), the K88 equivalent of RalI, the purpose of *ralI* is, likewise, unknown.

Another ORF, *faeJ*, is present in the K88 operon (3), but preliminary sequencing indicates there are no *fae* homologs downstream of *ralI*. However, there are one to two potential short ORFs followed by a third for which the sequence is incomplete (Fig. 1) but which demonstrates significant homology (49% amino acid identity) to the first 59 amino acids of the large (approximately 140 kDa) secreted Tsh (temperature-sensitive hemagglutination) protein of a pathogenic avian *E. coli*; this protein, in turn, resembles the EspC and SepA proteins of EPEC and *Shigella flexneri*, respectively (4, 32, 43). Roles for EspC and SepA have not been defined, but it is of interest that a homolog of a gene (*tsh*) which mediates hemagglutination of chicken erythrocytes is located near an operon that is involved in hemagglutination and other types of adherence of a REPEC strain.

Preliminary sequencing upstream of *ralC* suggests that the DNA from *ralC* to *ralI* encompasses the entire region of K88 homology in strain 83/39. Instead of the expected homology to

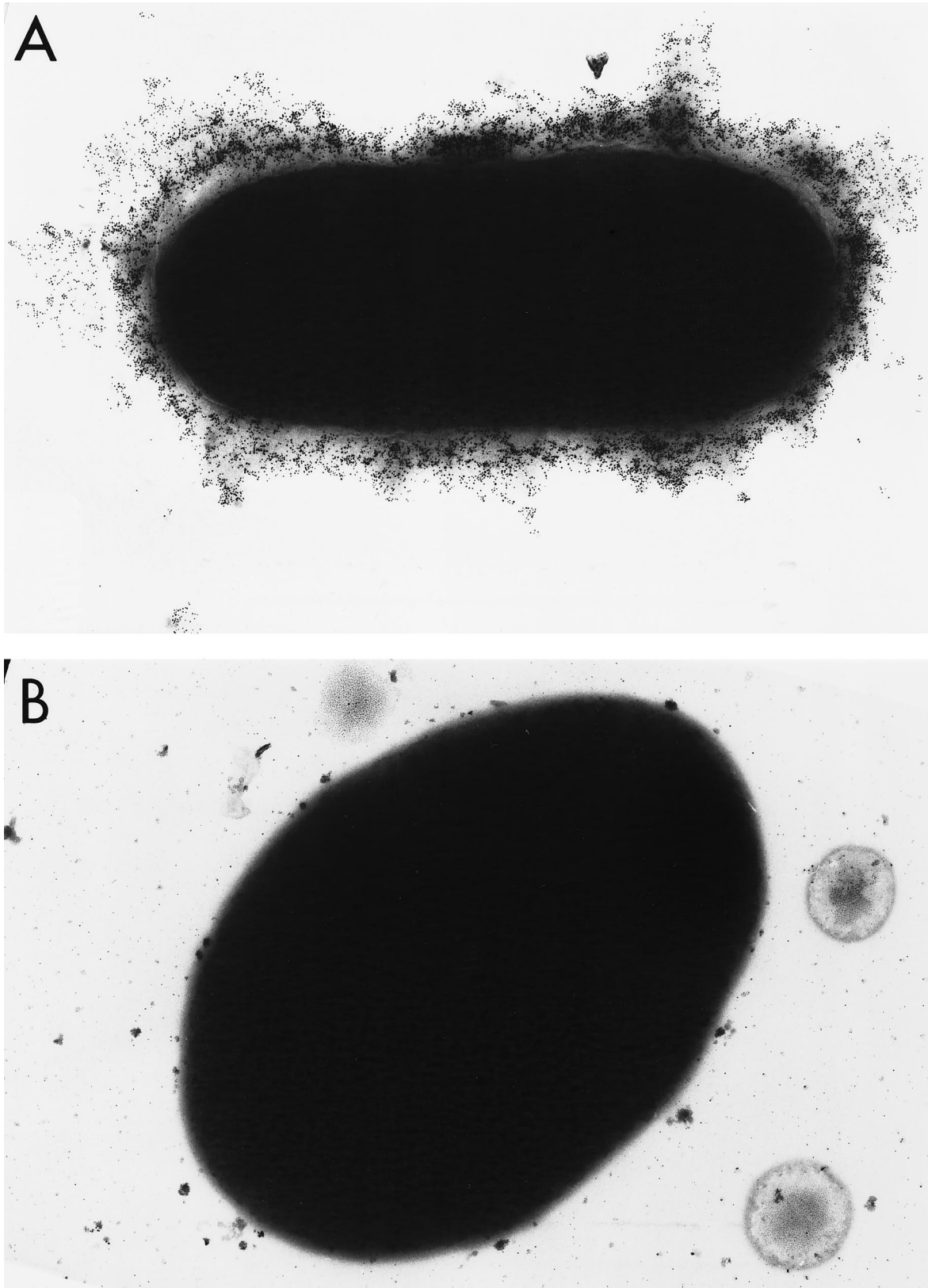


FIG. 4. (A) Immunoelectron microscopy of *E. coli* 83/39 showing binding of an adsorbed hyperimmune antiserum to a negatively staining network of fine fimbriae, as detected by anti-rabbit immunoglobulin G antibodies conjugated to 5-nm gold particles. (B) Electron micrograph of the *ralE* mutant, 83/39/23, showing a lack of the negatively staining fimbrial network surrounding the cell and, correspondingly, an absence of binding of the adsorbed antiserum as indicated by the absence of gold particles. Magnification: $\times 50,000$.

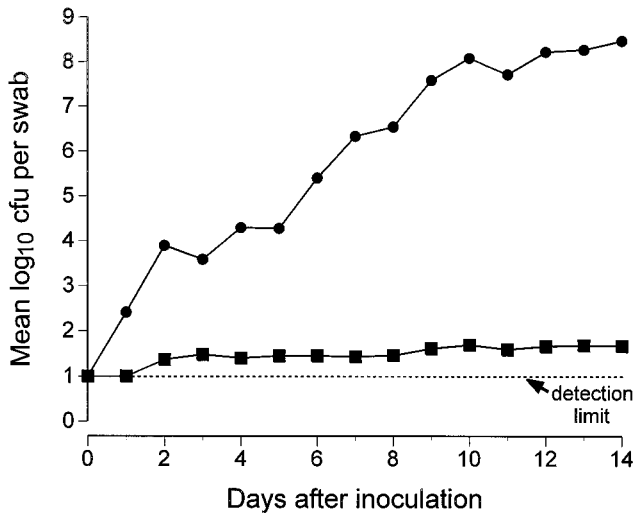


FIG. 5. Colonization of rabbits by REPEC 83/39 (●) and the *ralE* mutant, 83/39/23 (■), as measured by quantitative culture of rectal swabs. Data are the geometric mean CFU for five rabbits inoculated with 83/39 and the geometric mean CFU for 10 rabbits given 83/39/23. The detection limit of the culture method is indicated.

the K88 *faeB* gene, the ORF upstream from *ralC* displays homology to *afR* (1a), which is the proposed regulator of the *afR* operon responsible for the production of AF/R1 fimbriae by the REPEC strain RDEC-1 (GenBank accession no. L08467). Indications are that the *afR* homolog is transcribed in the opposite direction to the rest of the *ral* operon, which, since no promoters have been identified to date, implies that a promoter for the *ral* genes should lie upstream of *ralC* (Fig. 1).

The greatest conservation of deduced amino acid sequences between the ETEC and Ral proteins was in the chaperone genes, particularly in the periplasmic chaperones FaeE and RalE. Since these genes are also the regions of greatest homology between K88 and other fimbrial operons (16), this was to be expected. RalD and RalE also exhibited some of the

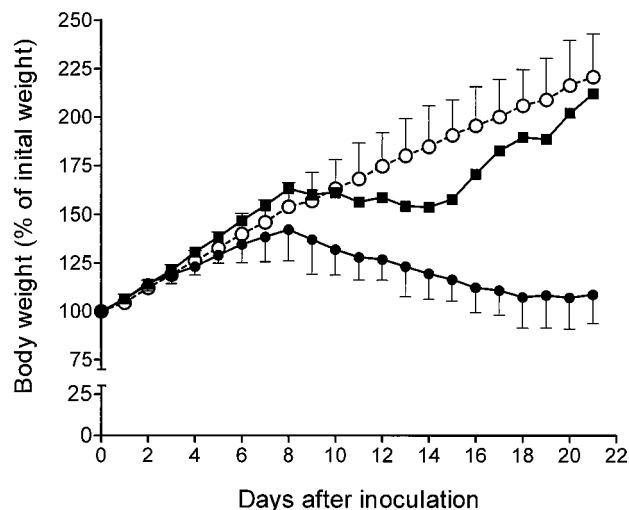


FIG. 6. Effect of infection with various REPEC strains on the body weight of rabbits. Data are the means \pm standard deviations of body weights (shown as a percentage of initial weight) of three diarrheic rabbits given 83/39 (●), one diarrheic rabbit colonized by 83/39/23 (■), and nine rabbits which were not colonized by this strain (○).

distinctive features of the Fae chaperones, including the N- and C-terminal cysteine residue pairs found in FaeD, and predicted signal cleavage sites which resulted in unusually long signal peptides similar in length to those of FaeD and FaeE (1b, 22, 47). Indeed, analysis by the method of von Heijne (33, 49) showed that all of the Ral proteins had predicted signal sequences which were comparable in length to those predicted for the corresponding Fae proteins (data not shown). Interestingly, however, it was in these N-terminal regions of the Ral proteins where, with the single exception of the putative major subunit, RalG, the homology to their Fae and Clp homologs was lowest. Thus, the percentage amino acid similarities within the proposed signal sequences were lower than those in the mature protein, while the reverse was true for RalG, where the signal sequence was the region of greatest conservation. Another N-terminal discrepancy between K88 and Ral was in the proposed GTG initiation codons of RalC and RalE, encoding valine rather than the usual methionine found in the Fae proteins. In the case of RalC, there are two valines encoded at the commencement of the gene, either of which could be the initiation codon, although the first one is in better proximity to the predicted ribosome binding site than the second. In addition, RalH has only one possible start codon rather than the three candidates of FaeH (3), and both *ralH* and *ralI* lack a consensus ribosome binding site, unlike their Fae counterparts. Thus, the differences within the intergenic regions increase towards the 3' end of the operon.

Of the Ral structural subunits, those most homologous to the ETEC operons are RalC and RalH, followed by RalF and RalI, with RalG, the major subunit, displaying the least homology. The adhesive capabilities of the K88 fimbriae are a property of the major subunit (2, 15). In K88 there are three antigenic variants of FaeG, termed K88ab, K88ac, and K88ad. They differ in their amino acid sequences and, consequently, the types of erythrocytes they agglutinate (2, 12, 27). Given the markedly different in vitro adherence phenotypes and species specificity of strain 83/39, it is logical that RalG should be appreciably dissimilar to FaeG. Since the K88ab variant was marginally more homologous to RalG than K88ac or K88ad, it was used for sequence comparisons.

The highest percentage amino acid identity was observed when *ral* was compared to the subunit genes of another REPEC operon, *afR2*. Since the adhesins encoded by these operons mediate binding to rabbit intestine, they would be expected to be more similar to one another than to the ETEC adhesins which bind to receptors in different hosts. The greatest homology between *ral* and *afR2* was observed in the minor subunit genes (H). The major subunits were more variable, particularly in the central region, which probably accounts for the different in vitro adherence phenotypes of the two strains (21, 38). It is probable that the *afR2* operon encodes homologs of all the *ral* genes, particularly since Fiederling et al. have reported that the smallest DNA fragment isolated which expressed functional AF/R2 is 10.1 kb (9). In addition, a nonadherent, attenuated mutant of REPEC B10 carries *TnphoA* upstream from the *afR2G* gene in a position equivalent to the N terminus of *ralE*, a mutation which we have shown to be attenuating.

The attenuation of the *ralE::TnphoA* mutant parallels the K88 system in which FaeE is required for stability of the major fimbrial subunit in the periplasm. Hence, *faeE* mutants do not produce K88 fimbriae (1b, 25). The observation that *faeF* mutants also produce fewer fimbriae and lose hemagglutinating ability (3, 25) correlates with the loss of adherence ability in mutant 83/39/51. Confirmation that the lack of adherence of the *ralE* and *ralF* mutants was attributable to the insertions in

ralE and *ralF* and not to polar effects of the transposon was demonstrated by the complementation of these mutations by pWin, a plasmid which supplied RalE and RalF in trans but not RalG since the gene is incomplete. These findings indicate that although the *ralE* and *ralF* mutants produce RalG, fimbriae are not assembled due to the absence of the chaperone or the RalF accessory subunit, respectively. This provides further evidence that RalE and RalF fulfill roles similar to those of their Fae counterparts.

The existence of *ral* homologs in B10 is not restricted to this strain since DNA hybridization experiments have indicated the presence of *ral* homologs on plasmids of other REPEC strains of different serotypes and adherence phenotypes (1a). Available sequence from one of these homologous operons indicates that there is a greater degree of genetic relatedness between the plasmid-encoded operons than between *ral* and the chromosomal *afv2*. Thus, the plasmid-encoded operons appear to be equivalent to the ab, ac, and ad variants of K88, whereas *afv2* is less closely related.

The protein detected in immunoblots of fimbrial extracts of strain 83/39 was 32 kDa, which is 3 kDa larger than the predicted molecular size of the RalG precursor protein and 5 kDa larger than the putative mature protein (approximately 27.2 kDa). Similar observations have been made with respect to several of the Fae proteins, the observed molecular weights of which are higher than those calculated from the gene sequences (1b, 3, 25). To confirm that the 32-kDa protein is the major fimbrial subunit and the product of *ralG*, however, purification and amino acid sequencing of the protein are required. It is also noteworthy that although the homologies of Ral to the K88 and CS31A operons were comparable, the morphology of the fimbriae of strain 83/39 most resembles that of CS31A, which has a capsule-like structure almost indistinguishable from that observed in this study (10).

The role of Ral in adherence and colonization was clearly demonstrated by the loss of adhesive ability of the Ral mutants and the difference between the colonizing ability and overall pathogenicity for rabbits of the *ralE* mutant and that of the wild type. These findings parallel those with respect to AF/R1 mutants of *E. coli* RDEC-1 and an AF/R2 mutant of B10, which displayed similar levels of attenuation for rabbits (30, 50).

In an earlier investigation, we found strain 83/39 to be more virulent than reported here (37). This may reflect the pronounced age-related susceptibility of rabbits to infection with REPEC and the fact that the rabbits in this experiment were 1 to 2 weeks older than those used previously. Alternatively, we may be witnessing a natural attenuation of 83/39 similar to that observed in RDEC-1, which, although it retains all known virulence factors, has inexplicably lost its high level of virulence for rabbits (reference 5 and unpublished data).

This is the first report of the complete nucleotide sequence of the structural genes of a fimbrial operon from a REPEC strain. The sequence has close structural and organizational homology to the gene sequence for K88 and CS31A fimbriae of ETEC strains from pigs and bovines, respectively. The contribution of the fimbriae encoded by these genes to adherence and virulence was demonstrated by an examination of mutant strains. The findings of this study provide confirmatory evidence that EPEC requires fimbria-mediated colonization of the intestinal tract to be fully virulent and suggest that the fimbriae which mediate this adherence are heterogeneous and, hence, may have originated from a variety of sources. This study also confirms the usefulness of the rabbit model as a means of investigating the pathogenesis of EPEC-induced dis-

ease and suggests possible control measures for EPEC based on fimbrial vaccines (39).

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